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RESEARCH ARTICLE

The transcarboxylase domain of pyruvate carboxylase is essential for assembly of the peroxisomal flavoenzyme alcohol oxidase

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pyruvate carboxylase; peroxisome; *Hansenula polymorpha*; alcohol oxidase; transposon mutagenesis; FAD.

Abstract

Pyruvate carboxylase (Pyc1p) has multiple functions in methylotrophic yeast species. Besides its function as an enzyme, Pyc1p is required for assembly of peroxisomal alcohol oxidase (AO). Hence, Pyc1p-deficient cells share aspartate auxotrophy (Asp^-) with a defect in growth on methanol as sole carbon source (Mut^-). To identify regions in *Hansenula polymorpha* Pyc1p that are required for the function of HpPyc1p in AO assembly, a series of random mutations was generated in the Hp*PYC1* gene by transposon mutagenesis. Upon introduction of 18 mutant genes into the *H. polymorpha PYC1* deletion strain (*pyc1*), four different phenotypes were obtained, namely Asp^- Mut^-, Asp^- Mut^+, Asp^+ Mut^-, and Asp^+ Mut^+. One mutant showed an Asp^+ Mut^- phenotype. This mutant produced HpPyc1p containing a pentapeptide insertion in the region that links the conserved N-terminal biotin carboxylation domain (BC) with the central transcarboxylation (TC) domain. Three mutants that were Asp^- Mut^- contained insertions in the TC domain, suggesting that this domain is important for both functions of Pyc1p. Analysis of a series of constructed C-terminal and N-terminal truncated versions of HpPyc1p showed that the TC domain of Pyc1p, including the region linking this domain to the BC domain, is essential for AO assembly.

Introduction

Peroxisomal matrix proteins are synthesized in the cytosol and posttranslationally imported into peroxisomes. Correct sorting requires peroxisomal targeting signals (PTS) that are recognized by one of the two known PTS receptor proteins, Pex5p or Pex7p. These receptors are soluble proteins that function as cycling receptors and may enter the peroxisomal matrix in complex with their cargo. For a recent review, see Heiland & Erdmann (2005).

Most peroxisomal matrix proteins contain a PTS1 sequence, which consists of three amino acids (SKL or conserved variants thereof), that is located at the extreme C-terminus (Heiland & Erdmann, 2005). The PTS1 is recognized by the C-terminal tetratricopeptide (TPR) repeat domain of Pex5p (Gatto *et al.*, 2000). The N-terminal part of Pex5p is involved in binding to the peroxisomal membrane (docking), the translocation process, and recycling to the cytosol (Otera *et al.*, 2002; Costa-Rodrigues *et al.*, 2004).

Interestingly, several examples exist of peroxisomal matrix proteins that are sorted to peroxisomes in a Pex5p-dependent way, but which either lack a PTS1 or contain a redundant PTS1. An example is *Saccharomyces cerevisiae* acyl-CoA oxidase. This peroxisomal flavoenzyme does not have a PTS1, but its sorting is dependent on ScPex5p. The association of acyl-CoA oxidase with ScPex5p does not require the C-terminal PTS1-binding domain of Pex5p, but a region in the ScPex5p N-terminus (Klein *et al.*, 2002; Schafer *et al.*, 2004). Alcohol oxidase (AO) of *Hansenula polymorpha* is an example of a peroxisomal protein that does contain a functional PTS1, which, however, is redundant for targeting of the protein to peroxisomes. Like acyl-CoA oxidase, AO is a flavoenzyme. We previously showed that import of AO can be mediated by a truncated Pex5p protein, which lacks the C-terminal TPR domain (Gunkel *et al.*, 2003), a phenomenon that has also been described for bakers' yeast acyl-CoA oxidase (Schafer *et al.*, 2004). Our previous data suggest that AO is only recognized by Pex5p after binding of its cofactor FAD (Evers *et al.*, 1994, 1996; Gunkel *et al.*, 2003). Hence, FAD binding may result in the formation of an as yet unknown PTS in AO (Gunkel *et al.*, 2003).

In *H. polymorpha* mutants defective in the cytosolic protein pyruvate carboxylase (HpPyc1p), newly synthesized AO monomers fail to bind FAD and, as a consequence, are

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not sorted to peroxisomes or assembled into enzymatically active octamers (Ozimek *et al*., 2003). The same phenomenon was observed in the related yeast *Pichia pastoris* (Ozimek *et al*., 2003). HpPyc1p is an anaplerotic enzyme that replenishes the tricarboxylic acid cycle with oxaloacetate generated from pyruvate. The function of HpPyc1p in AO assembly does not require enzymatically active HpPyc1p, but involves a novel, additional function of the protein (Ozimek *et al*., 2003). The fate of newly synthesized AO protein in *H. polymorpha* cells deleted for *PYC1* is similar to that observed in cells blocked in synthesis of the FAD (Evers *et al*., 1994), suggesting that HpPyc1p plays a role in binding FAD to AO monomers. In line with this assumption is the observation that HpPyc1p specifically interacts with AO (Ozimek *et al*., 2003).

How would HpPyc1p function in the FAD-binding process? One possibility is that HpPyc1p interacts with newly synthesized AO monomers to mediate or stabilize a protein conformation that is competent for FAD binding. Another possibility is that HpPyc1p is actively involved in donating FAD to AO, which implies that HpPyc1p (transiently) binds FAD.

In order to elucidate the molecular mechanisms involved in the novel function of HpPyc1p, we aimed to delineate the region(s) in HpPyc1p required to activate AO. An NCBI Conserved Domain Search (Marchler-Bauer *et al*., 2005) did not reveal any unique domain in HpPyc1p that is absent in Pyc proteins of nonmethylotrophic yeast species. Instead, like all other known eukaryotic Pyc proteins, HpPyc1p contains three functional domains, namely an N-terminal biotin carboxylation (BC) domain, a central transcarboxylation (TC) domain, and a C-terminal biotin carboxyl carrier (BCC) domain (Attwood, 1995). The BC domain is involved in ATP-dependent carboxylation of biotin. The TC domain transfers a carboxyl group from biotin to pyruvate, whereas the BCC domain binds biotin (Attwood & Wallace, 2002). None of these domains contains an FAD-binding motif. However, the BC domain contains an ATP-binding site, which might be capable of transiently binding the adenine nucleotide in FAD.

Using different mutagenesis approaches, we dissected the region in HpPyc1p required for AO assembly. The outcome of these studies is presented in this article.

Materials and methods

Organisms and growth conditions

The *H. polymorpha* strains used in this study were wild-type NCYC 495 leu1.1 (Gleeson & Sudbery, 1988), pyc1 leu1.1 (Ozimek *et al*., 2003), and *ass3–110* (van Dijk *et al*., 2002). Cells were cultivated at 37 °C in mineral media (Van Dijken *et al*., 1976), supplemented with 0.5% glucose, 0.5% methanol or a mixture of 0.1% glycerol and 0.5% methanol as carbon sources. As nitrogen source, 0.2% ammonium sulfate or 0.25% methylamine was used. Solid media contained 0.67% Yeast Nitrogen Base without amino acids (Difco, Sparks, MD), and were supplemented with 1% glucose (YND) or 0.5% methanol (YNM) and 2% agar. When needed, aspartate was added to a concentration of 60 mg L^{-1}. *Escherichia coli* DH5α was used for cloning purposes, and cultivated as described (Sambrook *et al*., 1989).

Genetic manipulations and yeast transformation

All standard DNA procedures were performed as previously described (Sambrook *et al*., 1989). Electrotransformation of *H. polymorpha* cells was carried out as detailed by Faber *et al*. (1994). The Hp*PYC1* gene of original mutant *ass3–110* (Ozimek *et al*., 2003) was sequenced after amplification by PCR using chromosomal DNA isolated from *ass3–110* cells, and primers PYC-ATG and PYC-STOP (Table 1).

Transposon mutagenesis of *H. polymorpha PYC1*

The *H. polymorpha PYC1* gene was cloned from pHIPX5-PYC1 (Ozimek *et al*., 2003) as an NcoI (Klenow filled-in)/SphI fragment into pHIPX6 (Kiel *et al*., 1995) digested with BamHI (Klenow filled-in) and SphI. The resulting plasmid, pHIPX6-PYC1, was subjected to NotI digestion followed by Klenow fill-in and religation to eliminate the NotI restriction site. The final construct was mutagenized with the transposon-based MGS mutation generation system (Finnzymes OY, Espoo, Finland). Plasmids were used to transform *E. coli*, and colonies with transposon-containing plasmids were selected on the basis of chloramphenicol resistance gene present in the transposon.

Further restriction analysis of the resulting plasmids with BamHI/HindIII allowed selection of mutants that carried transposons in the Hp*PYC1* gene, but not in the vector backbone. The insertion sites were sequenced using the

Table 1. Primers used in this study

Primer name	Sequence (5′–3′)
Entranceposon CAM reverse	CTACCGGAAGCAGTGTGACC
PYC1 trunc. 1	GGGATATCATGCAATCCAAGAACAGAGC
PYC1 trunc. 2	GGGATATCATGACCAGAGATGCTGACATCC
PYC1 trunc. 3	GGGATATCATGTGCTTGATCATGGACAC
PYC1-STOP	CCGCATGCGCAGAGCGAGACGC
PYC-ATG	CTTCCATGGCCCAGGTCG
SAK001	ATAGGGCGAATTGGGGATCGATC
SAK002	GCGGCCCGGGTTAACCATCGAAAGCCCTAATTTGG
SAK011	GCGCATGCTTAGTGGTGGTGGTGGTGGTGTCCAATGG CCATCACCTTGATGAC
SAK014	GGAAAGCGGCCGCATGGCCCAGGTCGAGGATTATTC
SAK015	GGGTTAGTCCAAGGCAGCAATGAACG

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Table 2. *Hansenula polymorpha* and *Escherichia coli* strains used in this study

Strain	Relevant properties	Reference
Wild type	*H. polymorpha* NCYC 495 *leu1.1*	Gleeson & Sudbery (1988)
pyc1	*H. polymorpha* NCYC 495 *pyc1 leu1.1*	Ozimek *et al.* (2003)
PYC1^{WT}	*pyc1* containing plasmid pHIPX6-PYC1	This study
PYC1$^{1,2\ldots-20}$	*pyc1* containing plasmids pHIPX6-PYC1$^{1,2\ldots-20}$	This study
PYC1$^{1-485(IM)}$	*H. polymorpha* NCYC 495 *ass3–110 leu1.1*, Initial Mutant	van Dijk *et al.* (2002)
PYC1$^{1-560}$	*pyc1* containing plasmid pSAK1-PYC1$^{1-560}$	This study
PYC1$^{1-815}$	*pyc1* containing plasmid pSAK1-PYC1$^{1-815}$	This study
PYC1$^{480-1061}$	*pyc1* containing plasmid pSAK4-PYC1$^{480-1061}$-HIS$_6$	This study
PYC1$^{480-1175}$	*pyc1* containing plasmid pHIPX6-PYC1$^{480-1175}$	This study
PYC1$^{512-1175}$	*pyc1* containing plasmid pHIPX6-PYC1$^{512-1175}$	This study
PYC1$^{561-1175}$	*pyc1* containing plasmid pHIPX6-PYC1$^{561-1175}$	This study
pyc1::P$_{AMO}$*PYC1*	*pyc1* with a one-copy integration of plasmid pHIPX5-PYC1	Ozimek *et al.* (2003)
E. coli SG13009(pREP4)	*E. coli* SG13009[pREP4] containing plasmid pQE60-PYC1	Ozimek *et al.* (2003)
E. coli SG13009(pREP4)	*E. coli* SG13009(pREP4) containing plasmid pQE60-PYC1^{13}	This study

primer 'entranceposon CAM reverse' (Table 1), corresponding to transposon positions 147–128. The main body of the transposon was cut out by NotI digestion, and plasmids were religated, resulting in plasmids that contained random insertions of 15 bp in the Hp*PYC1* gene. These plasmids were used to transform *H. polymorpha pyc1*. Transformants were screened for their ability to complement either of the two or both growth phenotypes of the *pyc1* strain, namely aspartate auxotrophy and inability to grow on methanol.

Construction of truncated Hp*PYC1* genes

Fragments of the Hp*PYC1* gene coding for truncated HpPyc1p proteins consisting of amino acids 480–1175 (HpPyc1$^{480-1175}$p), 512–1175 (HpPyc1$^{512-1175}$p) and 561–1175 (HpPyc1$^{561-1175}$p) were amplified using pBSK-PYC1 as template and the common reverse primer PYC1-STOP (Ozimek *et al.*, 2003) and primers PYC1 trunc.1, PYC 1 trunc.2 or PYC1 trunc.3, respectively (Table 1). To facilitate further cloning, PCR products of 2.2, 2.1 and 2.0 bp, respectively, were ligated into pBluescriptII KS(+) (Stratagene), digested with SmaI. Hp*PYC1* fragments were subsequently retrieved by digestion of the obtained plasmids with SmaI and SphI, and cloned into pHIPX6 (Kiel *et al.*, 1995), digested with BamHI (Klenow filled-in) and SphI. The resulting plasmids were designated pHIPX6-PYC1$^{480-1175}$, pHIPX6-PYC1$^{512-1175}$ and pHIPX6-PYC1$^{561-1175}$, and encoded the indicated truncated proteins under control of the P$_{PEX3}$ (Table 2).

A 3.0-kb Hp*PYC1* fragment coding for amino acids 1–560 of HpPyc1p was amplified by PCR using primers SAK001 and SAK002, and ligated as a 1.7-kb BamHI/SmaI fragment into a 7.9-kb fragment of pSAK1 that had been digested with the same enzymes. The resulting plasmid was designated pSAK1-PYC1$^{1-560}$. Vector pSAK1 was constructed by ligating a 1.5-kb Asp718i/HpaI pHS6A (Leao-Helder *et al.*, 2003) fragment into the 6.4-kb Asp718i/PvuII-digested fragment of pHIPX4-HNBESX (K.B. Rechinger, unpublished results). The 2.5-kb fragment coding for amino acids 1–815 of HpPyc1p was generated by PCR using primers SAK014 and SAK015, and cloned as a NotI-digested insert into the 7.9-kb NotI/SmaI-digested vector pSAK1. The plasmid was designated pSAK1-PYC1$^{1-815}$, and contained the truncated gene under control of the strong P$_{AOX}$ promoter.

A fragment coding for amino acids 480–1061 of HpPyc1p containing a C-terminal His$_6$-tag (under control of P$_{AMO}$) was obtained by PCR using primers PYC 1 trunc.1 and SAK011. The 1.8-kb product was digested with SphI and ligated into a 7.4-kb BamHI (Klenow filled-in)/SphI fragment of pSAK4. The resulting plasmid was designated pSAK4-PYC1$^{480-1061}$-His$_6$. Vector pSAK4 was cloned by ligation of a 1.8-kb Asp718i/NheI fragment of pSAK1 into the 7.3-kb fragment of pHIPX5-stuffer (M. Komori, unpublished results), digested with the same enzymes.

The plasmids were sequenced (Baseclear, Leiden, the Netherlands) and used to transform *H. polymorpha pyc1 leu1.1* cells, yielding strains PYC1$^{480-1175}$, PYC1$^{512-1175}$, PYC1$^{561-1175}$, PYC1$^{1-560}$, PYC1$^{1-815}$, and PYC1$^{480-1061}$, respectively.

Isolation of His$_6$-tagged versions of HpPyc1p and HpPyc1^{13}p

Construction of the plasmid pQE60-PYC1 for the expression of a C-terminally His$_6$-tagged HpPyc1p in *E. coli* has been described previously (Ozimek *et al.*, 2003). To obtain the analogous plasmid for expression of the His$_6$-tagged version of HpPyc1^{13}p, a BamHI/PstI fragment of the HpPYC1 gene (963 bp) in pQE60-PYC1 was replaced by the fragment of HpPYC1^{13} digested with the same enzymes (978 bp). The resulting plasmid pQE60-PYC1^{13} was used to transform *E. coli* SG13009(pREP4). Purification of both

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proteins was performed as previously described (Ozimek *et al.*, 2003).

AO/HpPyc1^{13}p binding studies

Sepharose beads containing covalently bound AO or bovine serum albumin (BSA) were prepared as described by Evers *et al.* (1993). Binding studies using purified HpPyc1^{13}p as well as HpPyc1p were performed as described previously (Ozimek *et al.*, 2003).

Fluorescence measurements

Fluorescence spectra were obtained using a Fluorolog 3.2.2 (Horiba Jobin Yvon) spectrofluorometer equipped with a thermostatically controlled cuvette holder. All measurements were performed at room temperature (22 °C) using purified His_6-tagged HpPyc1p and HpPyc1^{13}p in 25 mM Tris-HCl (pH 7.0) supplemented with 50 mM KCl and 1 mM dithiothreitol. The absorbance of the samples at 280 nm was kept below 0.1 to minimize the inner filter effect. Excitation and emission slits were kept at 2 nm. The spectrum of a blank solution, containing all components except the protein, was subtracted from each sample spectrum. All collected spectra were corrected for wavelength-dependent instrumental response characteristics.

Circular dichroism measurements

Circular dichroism (CD) measurements were performed using a Jasco J-715 spectropolarimeter, equipped with a Peltier temperature control system set at 20 °C. HpPyc1-His_6 and HpPyc1^{13}-His_6 proteins were dialyzed against 10 mM phosphate buffer (pH 7.0) prior to the measurements. Typical protein concentrations were 0.1–0.2 μM for far-UV CD measurements in a 0.1-cm cuvette and for near-UV CD measurements in a 1-cm cuvette. The scan speed was set at 10 and 100 nm min^{-1} for measurements in the far-UV and near-UV regions, respectively. Ten scans were averaged for each sample during data collection in the far-UV region, and 30 scans in the near-UV region. The spectral bandwidth was 1.0 nm. The response time was 2 s during the measurements in the far-UV, and 0.5 s in the near-UV. The blank (10 mM phosphate buffer, pH 7.0) recorded under the same conditions was subtracted from the corresponding CD spectra. The analysis of far-UV CD spectra for secondary structure content was performed using CDNN version 2 software (Bohm *et al.*, 1992).

Biochemical methods

AO activity was measured as described by Verduyn *et al.* (1984) in crude extracts, prepared according to van der Klei *et al.* (1991). AO monomers and octamers were separated by velocity centrifugation in sucrose density gradients (Goodman *et al.*, 1984). Protein concentrations were determined using the Bio-Rad assay kit (Bio-Rad Gmbh, Munich, Germany), using BSA as a standard. SDS-PAGE (Laemmli, 1970) and Western blotting were performed as previously described (Kyhse-Andersen, 1984). Blots were probed with antisera against *H. polymorpha* AO or HpPyc1p.

Results

Transposon mutagenesis of HpPYC1

In order to identify regions in HpPyc1p specifically required for AO assembly, mutants of the Hp*PYC1* gene were created using transposon mutagenesis and subsequently analyzed upon expression in an *H. polymorpha PYC1* deletion strain (pyc1). In order to mutagenize Hp*PYC1*, a self-replicating plasmid was constructed, pHIPX6-*PYC1*, containing the Hp*PYC1* gene under control of the *PEX3* promoter (Kiel *et al.*, 1995). As shown in Fig. 1, introduction of this plasmid into *pyc1* cells resulted in functional complementation of pyc1 cells, which are auxotrophic for aspartate (Asp$^-$), due to the absence of Pyc1p enzyme activity, and unable to utilize methanol (Mut$^-$) because of the defect in AO assembly (Ozimek *et al.*, 2003).

After transposon mutagenesis, 180 plasmids containing the transposon were further analyzed. Of these, 20 plasmids were selected that contained a single transposon inserted in the Hp*PYC1* gene, but not in the vector backbone. From these plasmids, corresponding constructs were obtained that contained 15 base pair insertions in the Hp*PYC1* gene (see 'Materials and methods'). The amino acid sequences of the pentapeptide insertions as well as the position of these insertions in HpPyc1p are presented in Table 3 and Fig. 2. These data show that insertions are distributed over the entire protein. Only in two cases were identical mutants obtained (Table 3 and Fig. 2: mutants 1 and 2, and mutants 7 and 8).

The plasmids were introduced into the *H. polymorpha PYC1* deletion strain (*pyc1*), and transformants were analyzed for aspartate auxotrophy and the capacity of the cells to grow on methanol (Table 3). Eleven out of 20 mutant genes fully complemented both defects of *H. polymorpha pyc1* cells (i.e. resulted in an Asp$^+$ Mut$^+$ phenotype). Four transformants were Asp$^-$ Mut$^-$, indicating that pentapeptide insertions had occurred that affected both activities of HpPyc1p. Two transformants showed normal growth on methanol (Mut$^+$) but an intermediate phenotype with respect to aspartate auxotrophy (Asp$^{+/-}$), reflected by a significantly delayed appearance of colonies on solid glucose media without aspartate. Two transformants showed an Asp$^-$ Mut$^+$ phenotype. These mutations, which are both localized in the BC domain, apparently affect the HpPyc1p enzyme activity but not its function in AO assembly.

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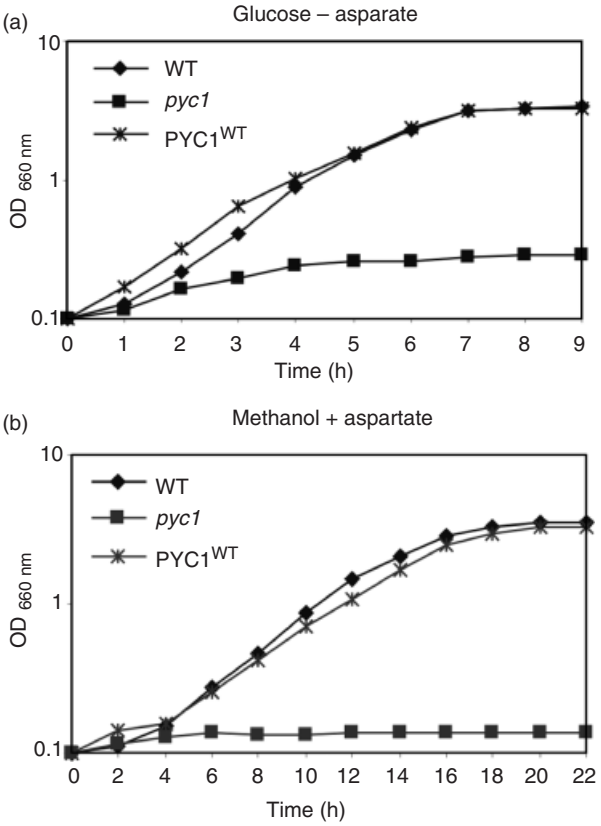

Fig. 1. Complementation of *Hansenula polymorpha* pyc1 cells by pHIPX6-PYC1. HpPYC1 expressed from plasmid pHIPX6, under control of the PEX3 promoter, complements the Mut$^-$ Asp$^-$ phenotype of *Hansenula polymorpha* pyc1 cells. Cells were grown in liquid media containing glucose without aspartate (a) or methanol plus aspartate (b). WT, wild-type cells containing the empty vector pHIPX6; pyc1, pyc1 cells containing the empty vector; PYC1^{WT}, pyc1 strain expressing wild-type HpPYC1 from pHIPX6-PYC1. Growth is expressed as OD at 660 nm ($OD_{660\,nm}$).

However, one transformant (strain PYC1^{13}) was obtained that was Asp$^+$ Mut$^-$, which points to a specific defect in AO assembly.

These data demonstrate that the enzymatic function of HpPyc1p and its function in AO assembly can be separated.

Analysis of strains PYC1^3, PYC1^{14}, PYC1^{15} and PYC1^{16}

Four transformants (strains PYC1^3, PYC1^{14}, PYC1^{15} and PYC1^{16}; Fig. 2 and Table 3) showed an Asp$^-$ Mut$^-$ phenotype, suggesting that the regions altered in the HpPyc1p proteins in these strains are important for both HpPyc1p enzyme activity and its function in AO assembly. In PYC1^3, the pentapeptide insertion occurred at the N-terminus. The other three (PYC1^{14}, PYC1^{15} and PYC1^{16}) all contained the insertion in the TC domain.

Table 3. Mutant HpPyc1p proteins obtained by pentapeptide insertions

Mutant phenotype	Mutant number	Position and sequence of the pentapeptide insertion
Asp$^+$, Mut$^+$	1, 2	21 LRPHL
	4	52 IAAAA
	5	123 CGRNS
	6	124 CGRSG
	7, 8	139 VRPHG
	9	248 CGRIH
	10	353 CGRMQ
	19	1106 MRPHP
	20	1163 SAAAD
Asp$^{+/-}$, Mut$^+$	17	901 CGRIK
	18	1020 VRPHQ
Asp$^-$, Mut$^+$	11	390 CGRTG
	12	434 VRPHR
Asp$^+$, Mut$^-$	13	541 LRPHR
Asp$^-$, Mut$^-$	3	34 CGRSE
	14	670 DAAAV
	15	754 SAAAI
	16	794 CGRNC

Sequences of the pentapeptide insertions obtained by transposon mutagenesis with the corresponding phenotype of *pyc1* cells producing the different mutant HpPyc1p proteins. The phenotypes were determined based on the appearance of colonies on solid media relative to *pyc1* cells harboring the empty vector (*pyc1*) and *pyc1* cells expressing Hp*PYC1* from pHIPX6-PYC1 (PYC1^{WT}). Mut$^+$ and Asp$^+$ strains grew like PYC1^{WT} controls on media containing methanol/aspartate or glucose without aspartate, respectively (checked after 2 days of incubation). Strains were classified Mut$^-$ or Asp$^-$ when no colonies had appeared after 7 days of incubation on media containing methanol/aspartate or glucose without aspartate, respectively. Strains were scored as Asp$^{+/-}$ when, on glucose media lacking aspartate, no colonies were observed after 2 days of incubation, but had appeared after 4 days of incubation.

To test whether the insertions that occurred in these strains affected the HpPyc1p protein levels, the HpPyc1p levels in these strains were analyzed by Western blotting using anti-HpPyc1p antibodies. As shown in Fig. 3, each of the Asp$^-$ Mut$^-$ mutant strains produced HpPyc1p protein, although the levels were reduced relative to the control strain producing wild-type HpPyc1p. The lowest amounts of HpPyc1p were detected in strains PYC1^3 and PYC1^{14}. Enzyme activity measurements revealed that neither of these strains showed AO activities that were higher than those observed in *pyc1* control cells [ranging from 0.02 to 0.08 U mg^{-1} protein (Ozimek *et al.*, 2003); Fig. 3].

Using a strain containing the Hp*PYC1* gene under control of the P_{AMO} promoter (*H. polymorpha pyc1*::P_{AMO}*PYC1*), we previously showed that strongly reduced HpPyc1p levels are sufficient to activate AO (Ozimek *et al.*, 2003). We cultivated cells of this strain at conditions that resulted in comparable HpPyc1p levels as observed in the four Asp$^-$ Mut$^-$ strains. At these levels of HpPyc1^{WT}p, AO activities of 0.41 U mg^{-1} were observed, indicating that the amount of HpPyc1p was

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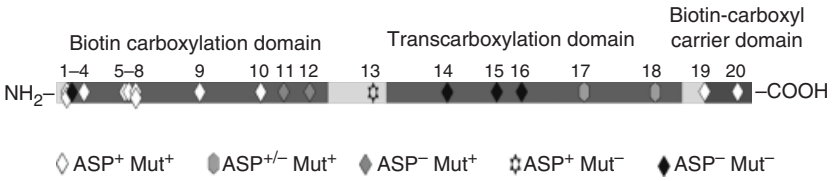

Fig. 2. Transposon mutagenesis of Hp*PYC1*. Schematic overview of the phenotypes (shown by different symbols) of *Hansenula polymorpha* cells producing mutant HpPyc1p proteins. Dark gray boxes represent the domains of HpPyc1p based on homology with related enzymes (source: NCBI Conserved Domain Search). The numbers correspond to the strain numbers indicated in Table 3.

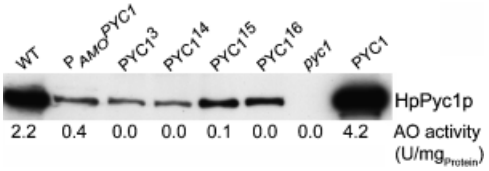

Fig. 3. HpPyc1p protein levels and AO enzyme activities in cells of strains PYC1[3], PYC1[14], PYC1[15] and PYC1[16]. Cells of the mutant strains as well as *pyc1* and PYC1[WT] controls were grown for 17 h on glycerol/methanol media supplemented with aspartate. Cell extracts were analysed by Western blotting using antibodies against HpPyc1p. The HpPyc1p levels in cells of the mutant strains were compared with that in cells of a strain producing artificially reduced levels of wild-type HpPyc1p (P_{AMO}*PYC1*), which were grown on glycerol/methanol/aspartate media in the presence of ammonium sulfate to repress P_{AMO}. The artificially reduced levels of wild-type HpPyc1p still resulted in AO assembly (0.41 vs. 0.02 U mg^{-1} protein observed in *pyc1* control cells). The AO enzyme activities in cells of strains PYC1[3], PYC1[14], PYC1[15] and PYC1[16] were in the same range as observed in the *pyc1* control cells. For the Western blot, equal amounts of protein were loaded per lane. To visualize the low HpPyc1p levels in cells of the mutant strains, relatively long exposure times were used.

sufficient for AO assembly (Fig. 3). In cells of the PYC1[15] and PYC1[16] strains, the HpPyc1p levels were at least as high as in the *pyc1*::P_{AMO}*PYC1* cells. On the basis of these data, we conclude that the defect in AO assembly in PYC1[15] and PYC1[16] is not due to limiting HpPyc1p levels.

PYC1[13] is specifically affected in AO assembly

The phenotype of mutant PYC1[13] (Asp$^+$ Mut$^-$) suggests a specific defect in AO assembly. This was supported by detailed growth experiments (Fig. 4), which revealed that PYC1[13] cells grew on glucose-containing media that lacked aspartate, as did the wild-type control strain (designated PYC1[WT]). Moreover, PYC1[13] cells failed to grow on methanol, like *H. polymorpha pyc1* cells. Western blot analysis revealed that HpPyc1[13] protein levels were slightly reduced in comparison to the PYC1[WT] control (Fig. 5a). Enzyme activity measurements demonstrated that the specific AO activities in cell-free extracts of PYC1[13] cells (0.06 U mg^{-1} protein) were very low, as in *pyc1* cells, confirming the AO assembly defect in PYC1[13] cells.

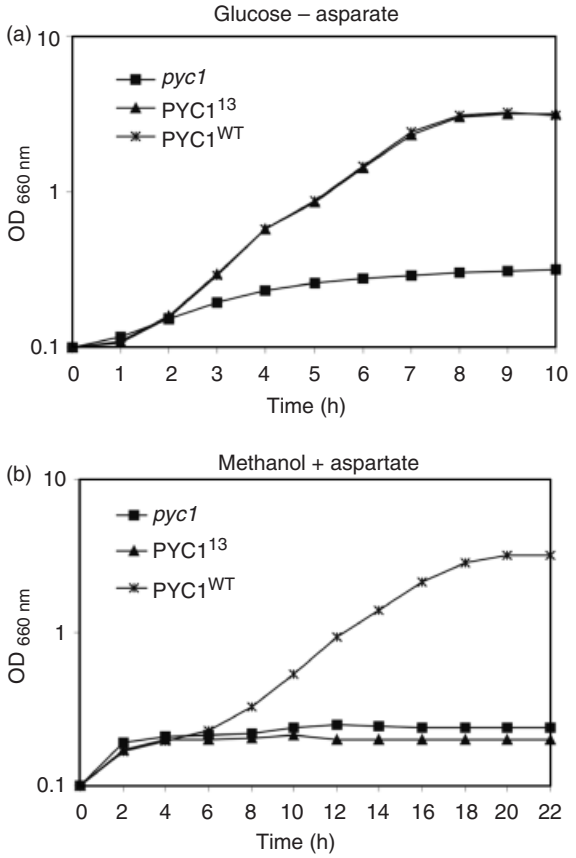

Fig. 4. HpPyc1[13]p is enzymatically active but is unable to mediate AO assembly. Cells were grown in mineral medium containing either glucose without aspartate (a) or methanol plus aspartate (b). *pyc1*, *pyc1* cells containing the empty vector pHIPX6; PYC1[WT], *pyc1* cells containing pHIPX6 with the Hp*PYC1* gene; PYC1[13], *pyc1* cells expressing mutant gene Hp*PYC1*[13] from plasmid pHIPX6. PYC1[13] cells normally grow on glucose media lacking aspartate, indicating that HpPyc1[13]p is enzymatically active. However, PYC1[13] cells fail to grow in methanol media (Fig. 4b), which points to a defect in AO assembly. Growth is expressed as OD measured at 660 nm ($OD_{660\,nm}$).

Next, we analyzed the oligomeric state of AO protein in PYC1[13]. In *pyc1* cells, AO monomers accumulate, whereas in wild-type cells, the majority of the AO protein is octameric (Ozimek *et al.*, 2003). Western blot analysis of fractions

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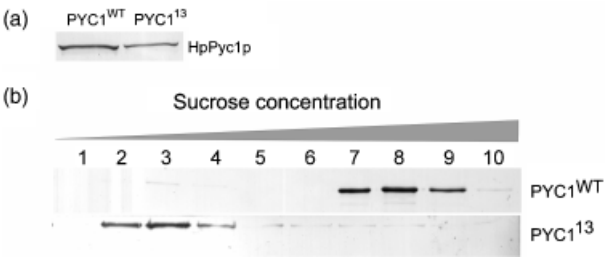

Fig. 5. PYC1^{13} cells accumulate monomeric AO. (a) Western blot analysis of total cell extracts using antibodies against HpPyc1p showed that the levels of HpPyc1^{13}p synthesized in PYC1^{13} cells are slightly lower as compared to PYC1^{WT} controls. Equal amounts of proteins were loaded per lane. (b) Analysis of the oligomeric state of AO in cell-free extracts. In PYC1^{13} cells, AO protein is predominantly present in the monomeric form (fractions 2–4), whereas the bulk of the AO protein in extracts of PYC1^{WT} cells localizes to fractions 7–9, where AO octamers sediment. Cells were grown for 13 h in liquid media containing 0.1% glycerol (a) and 0.5% methanol (b).

obtained after sucrose density centrifugation (Fig. 5b) showed that the majority of AO protein in PYC1^{13} cells sedimented to fractions 2, 3 and 4, which correspond to monomeric AO (Goodman *et al.*, 1984), whereas in PYC1^{WT}, AO was present in fractions 7, 8 and 9, the position of AO octamers (Goodman *et al.*, 1984). Immunocytochemical analysis revealed that in PYC1^{13} cells, AO protein was mislocalized to the cytosol, as was observed for *pyc1* cells (data not shown; Ozimek *et al.*, 2003).

Taken together, these data demonstrate that the pentapeptide insertion that had occurred in HpPyc1^{13}p abolished the function of the protein in AO assembly. Because PYC1^{13} cells are not auxotrophic for aspartate, HpPyc1^{13}p is apparently still enzymatically active.

HpPyc1^{13}p and AO physically interact

Previously, we have shown that HpPyc1p physically interacts with AO protein (Ozimek *et al.*, 2003). We were therefore interested in determining whether the pentapeptide insertion in HpPyc1^{13}p influenced the affinity of the protein for AO. To this end, His_6-tagged versions of both Pyc1 proteins were produced in *E. coli* and affinity purified. The purified HpPyc1p and HpPyc1^{13}p were loaded onto columns containing immobilized AO protein. Figure 6 shows Western blots prepared from the fractions collected in a typical binding assay. The data indicate that – like HpPyc1p – HpPyc1^{13}p is able to interact with AO protein, as significant amounts of HpPyc1^{13}p protein were detectable in the elution fraction (E). No HpPyc1^{13}p protein was detected in the elution fraction obtained from a BSA-containing column that was used as a control. Strikingly, only part of the loaded HpPyc1^{13}p (Input) was recovered in the flow-through (F) and elution (E) fractions, which was not

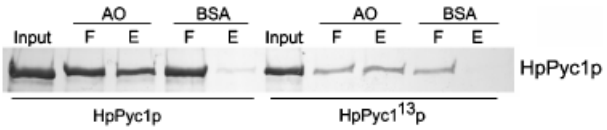

Fig. 6. HpPyc1^{13}p has retained its capacity to physically interact with AO protein. Purified HpPyc1p and HpPyc1^{13}p (Input) were loaded onto columns containing immobilized AO or BSA, used as a control. The columns were washed with 20 column volumes of wash buffer (F, flow through). Bound proteins were eluted using a buffer containing 8 M urea (E, elution). Equal portions of Input, F and E were subjected to SDS-PAGE and analyzed by Western blotting. Blots were probed with anti-HpPyc1p antibodies. Using wild-type HpPyc1p (left panel), a significant portion of the protein was recovered in fraction E, whereas no HpPyc1p was found in the elution fractions (E) using BSA-containing control columns. Essentially the same result was obtained using mutant HpPyc1^{13}p (right panel), with the exception that less protein was recovered in the E and F fractions, probably due to protein instability.

observed in the control experiment using wild-type HpPyc1p, suggesting that purified HpPyc1^{13}p is relatively unstable *in vitro* or forms aggregates that are not eluted from the column.

Fluorescence and CD analysis

To analyze whether the mutation present in HpPyc1^{13}p caused major changes in the protein conformation, we used biophysical approaches, namely tryptophan (Trp) fluorescence analysis and CD.

The fluorescence of Trp residues is sensitive to local environmental changes that may be caused by changes in the tertiary structure of a protein. These changes can result in a shift of the spectral maximum of fluorescence emission, as well as in the Trp fluorescence quantum yield (Lakowicz, 1999). HpPyc1p and HpPyc1^{13}p contain eight Trp residues. Fluorescence emission spectra were obtained from purified HpPyc1p and HpPyc1^{13}p using excitation at 295 nm (Fig. 7). The spectrum of HpPyc1p shows a fluorescence emission maximum at 327 nm, which indicates that most Trp residues are buried in the hydrophobic interior of the protein. Purified HpPyc1^{13}p, however, shows a maximum at 333 nm, in conjunction with a decrease in fluorescence intensity of about 20% relative to the spectrum of wild-type HpPyc1p. The shift of the fluorescence maximum and the drop in quantum yield indicated that the environment of some of the Trp residues in HpPyc1^{13}p had become less hydrophobic.

Furthermore, the secondary and tertiary structure of both HpPyc1 proteins were analyzed by CD spectroscopy. Highly similar far-UV spectra were observed for both HpPyc1p and HpPyc1^{13}p (data not shown), indicating that the mutation caused no major changes in the secondary structure of the protein. The near-UV spectra (not shown) indicated a slight difference between the two proteins and supported the

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fluorescence results, implying that Trp residues are repositioned in the mutant protein to a more polar environment.

Taking these data together, we can conclude that the mutation in HpPyc1^{13}p slightly affects the tertiary structure but has no major effect on the secondary structure of the protein. Possibly, the mutant protein has a more open conformation, resulting in more polar environments of some of the Trp residues.

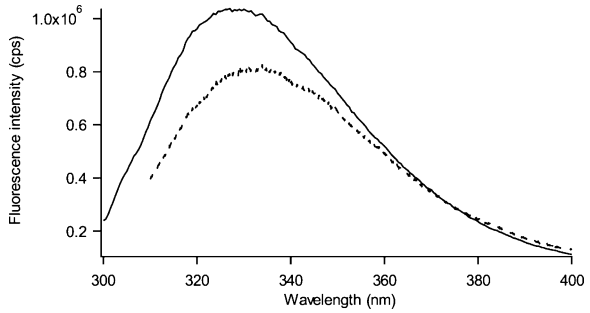

Fig. 7. Fluorescence analysis of the tertiary structure of HpPyc1^{13}p. Fluorescence emission spectra of HpPyc1p (solid line) and HpPyc1^{13}p (dashed line) excited at 295 nm. The observed shift of the maximum of Trp fluorescence emission of HpPyc1^{13}p towards longer wavelengths, as well as the lower fluorescence intensity of the mutant relative to wild-type HpPyc1p, indicate a minor change in the Trp environment. Spectra were normalized on the basis of protein concentrations.

The TC domain is important for the function of HpPyc1p in AO assembly

In order to further dissect the region in HpPyc1p that is important for AO assembly, we constructed a series of truncated genes encoding HpPyc1p variants containing different N-terminal and C-terminal deletions. In addition, we analyzed the mutation present in the original Hp*PYC1* mutant (*ass3–110*), which showed a defect in AO assembly (Ozimek *et al*., 2003).

Sequencing of the Hp*PYC1* gene of mutant *ass3–110* revealed a substitution of cytosine at position 1456 with thymine that leads to the introduction of a stop codon [Fig. 8a; HpPyc1$^{1-485(IM)}$]. Western blot analysis confirmed the presence of a truncated HpPyc1p form of the expected molecular mass (*c.* 53 kDa; Fig. 8b). This N-terminal portion of HpPyc1p contains the complete BC domain plus a small part of the region linking the BC and TC domains (Fig. 8a). Because this strain is unable to grow on methanol and showed an AO assembly defect (Table 4), the BC domain of HpPyc1p is apparently not able to activate AO.

In mutant PYC1^{13}, a mutation had occurred in the region between the BC and TC domains. To find whether this part of the protein was sufficient for AO assembly, we tested a construct encoding the BC domain plus the linking domain (HpPyc1$^{1-560}$p) or a larger region including also part of the TC domain (HpPyc1$^{1-815}$p). This resulted in the production of the expected truncated proteins in *pyc1* cells, as

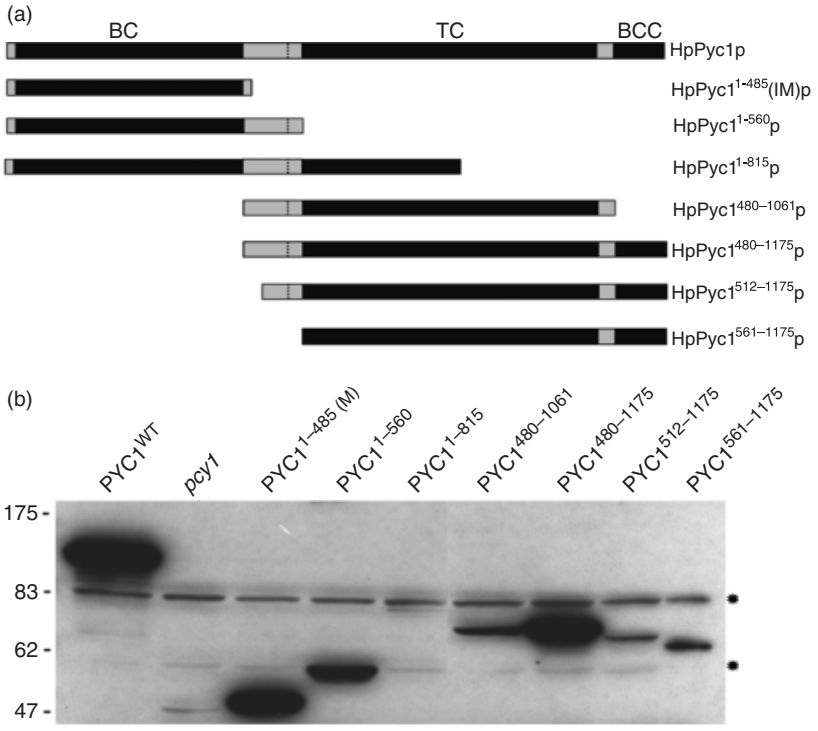

Fig. 8. The N-terminal BC domain and the C-terminal BCC domain are not required for AO assembly. (a) Schematic representation of truncated versions of HpPyc1p. Conserved domains known to be involved in HpPyc1p enzyme activity are indicated in black; BC, biotin carboxylation domain; TC, transcarboxylation domain; BCC, biotin carboxyl carrier domain. The dotted line represents the position of the pentapeptide insertion in HpPyc1^{13}p. HpPyc1$^{1-485\,(IM)}$p is the truncated protein that is produced by *Hansenula polymorpha ass3–110*. PYC1$^{480-1175}$ was engineered on the basis of the results of Sueda *et al*. (2004), who produced an analogous, stable TC–BCC fragment of Pycp from *Bacillus thermodenitrificans*. (b) Western blot analysis of total cell extracts of *Hansenula polymorpha* strains producing truncated forms of HpPyc1p, which are indicated in (a). The blot was probed with anti-HpPyc1p antibodies. The protein bands indicated with an asterisk represent nonspecific cross-reacting bands. Equal amounts of protein were loaded per lane. To visualize the low levels of the truncated HpPyc1p proteins, relatively long exposure times were used.

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Table 4. Growth properties and specific AO activities in different *Hansenula polymorpha* strains

Strain	$OD_{660\,nm}$	AO activity (U mg^{-1} protein)
$PYC1^{WT}$	3.4	3.83
pyc1	0.5	0.02
ass3–110 [$PYC1^{1–485\ (IM)}$]	0.6	0.09
$PYC1^{1–560}$	0.5	0.10
$PYC1^{1–815}$	0.6	0.00
$PYC1^{480–1061}$	2.8	1.59
$PYC1^{480–1175}$	1.9	1.57
$PYC1^{512–1175}$	0.6	0.05
$PYC1^{561–1175}$	0.5	0.11

Strains of *H. polymorpha* producing different truncated versions of Pyc1p were grown in mineral media containing methanol as sole carbon and energy source, in the presence of aspartate. The final OD (expressed as OD at 660 nm) was measured after 40 h of incubation. For AO enzyme activity measurements, cell extracts were prepared from cells grown for 24 h on a mixture of methanol and glycerol in the presence of aspartate.

demonstrated by Western blotting (Fig. 8b). However, the levels of $HpPyc1^{1–815}$p were very low, despite the fact that P_{AOX} was used. Growth experiments revealed that cells of both strains failed to grow on methanol plus aspartate, and also did not show enhanced AO enzyme activity relative to *pyc1* control cells (Table 4). Hence, the region between the BC and TC domains alone is not sufficient for AO assembly.

We next analyzed an N-terminal truncated HpPyc1p, lacking the entire BC domain ($HpPyc1^{480–1175}$p). Synthesis of this protein in *H. polymorpha pyc1* cells complemented the methanol growth defect paralleled by AO assembly (Table 4). On the basis of these data, we conclude that the BC domain is redundant for the function of HpPyc1p in AO assembly.

We also studied the importance of the BCC domain. The pentapeptide insertions in this region did not affect methanol growth (Fig. 2). As shown in Table 4, *pyc1* cells producing $HpPyc1^{480–1061}$p were able to grow on methanol and showed AO enzyme activity; hence, like the BC domain, the BCC domain is redundant for AO assembly.

To further investigate the role of the region between the BC and TC domains, we finally tested two constructs that contained the TC domain without the linking region ($HpPyc1^{561–1175}$p) or with only a portion of the linking region ($HpPyc1^{512–1175}$p). Both proteins were produced and present at the expected molecular mass (Fig. 8b). *Hansenula polymorpha pyc1* cells producing these truncated versions of HpPyc1p did not grow on methanol, and showed AO enzyme activities similar to that detected in *pyc1* control cells (Table 4). This led us to conclude that in addition to the TC domain, the linking region between the BC and TC domains is important for AO assembly.

Discussion

In this study, we aimed to delineate the region in *H. polymorpha* pyruvate carboxylase (HpPyc1p) that is required for the function of HpPyc1p in assembly of peroxisomal AO.

We used a transposon mutagenesis approach that has been successfully used before by others (Hallet *et al.*, 1997; Fransen *et al.*, 2005). There are multiple advantages of this approach, such as: (1) random distribution of mutations; (2) the possibility of selecting mutant genes containing one insertion per gene; (3) easy selection of mutated genes; (4) more severe effects than are obtained by classical PCR-based mutagenesis, because of the insertion of a pentapeptide; and (5) no introduction of frameshifts or stop codons.

The mutagenized Hp*PYC1* genes were transformed to *H. polymorpha pyc1* cells that are Asp^{-} Mut^{-}. Approximately half of the transformants showed a wild-type phenotype (Asp^{+} Mut^{+}). Four transformants were Asp^{-} Mut^{-}. In three of them (numbers 14, 15 and 16), insertions had occurred in the central TC domain. These strains contain similar (14) or higher (15 and 16) HpPyc1p protein levels than are required for AO assembly. Therefore, the defect in AO assembly is not due to too low HpPyc1p protein levels. Hence, the TC domain is most likely important for AO assembly, in addition to its function in the transfer of a carboxyl group from biotin to pyruvate (Attwood & Wallace, 2002). Two strains ($PYC1^{17}$ and $PYC1^{18}$) carrying pentapeptide insertions in the C-terminal part of the TC domain had only a minor effect on the ability of the cells to grow in the absence of aspartate, whereas no defect in methanol growth was detected. These mutations, therefore, most likely did not significantly disturb the function of the TC domain of HpPyc1p.

The data from the transposon mutagenesis studies pointed to a dispensable role of the N-terminal BC domain and the C-terminal BCC domain of HpPyc1p. Most insertions that occurred in these regions had no effect on the capacity of the cells to grow on methanol. In line with this, deletion of neither the BC domain nor the BCC domain affected methanol growth.

The BC domain of Pycp proteins contains an ATP-grasp fold (Galperin & Koonin, 1997). Our finding that the BC domain is not necessary for AO assembly suggests that it is unlikely that HpPyc1p is directly involved in donating FAD to AO, because the BC domain is the only domain in HpPyc1p that might be capable of binding FAD. Also, the function of HpPyc1p in AO assembly apparently does not require ATP. This excludes a role of HpPyc1p as a classical chaperone protein like Hsp60 or Hsp70, which are ATPases (Hartl & Hayer-Hartl, 2002).

The BC and TC domains in Pycp proteins are separated by a region of 80 amino acids. A pentapeptide insertion that

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Fig. 9. The region linking the BC and TC domains in HpPyc1p. Alignment of the region linking the BC and TC domains in HpPyc1p (residues 480–560) with the corresponding regions in Pyc proteins from other eukaryotes. Arrows indicate the N-termini of the truncated proteins HpPyc1$^{480–1175}$p (black arrow) and HpPyc1$^{512–1175}$p (gray arrow). The asterisk indicates the site of the pentapeptide insertion in HpPyc1^{13}p. 1, *Hansenula polymorpha* (Hp); 2, *Pichia pastoris* (Pp); 3, 4, *Saccharomyces cerevisiae* (Sc); 5, *Kluyveromyces lactis* (Kl); 6, *Yarrowia lipolytica* (Yl); 7, *Aspergillus niger* (An); 8, *Schizosaccharomyces pombe* (Sp); 9, *Drosophila melanogaster* (Dm); 10, *Homo sapiens* (Hs). Residues conserved in all presented sequences are indicated in black, those conserved in 80% of sequences are in dark gray, and those conserved in 60% of all sequences are in light gray.

occurred in this region (Pyc1^{13}) did not result in aspartate auxotrophy, but fully abolished its function in AO assembly, suggesting an important role for this region in AO assembly. In line with this conclusion is the finding that the N-terminal truncated proteins that lacked this domain completely (561–1175) or partially (512–1175) were not able to mediate AO assembly.

Alignment of the region that links the BC and TC domains (amino acids 480–560 in HpPyc1p; Fig. 9) with Pycp proteins from other eukaryotes revealed a highly conserved part (amino acids 480–511) followed by a less conserved region (amino acids 512–560). The pentapeptide insertion that occurred in HpPyc1^{13}p is present in the middle of the less conserved region (Fig. 9). Our studies suggest that this less conserved region in HpPyc1p may be the only region that is specifically involved in AO assembly, but not required for HpPyc1p enzyme activity.

Unfortunately, so far no three-dimensional structure of a eukaryotic Pyc protein is available. However, a three-dimensional structure is known of the 5S subunit of transcarboxylase from *Propionibacterium shermani* (Hall *et al.*, 2004), which is homologous to the HpPyc1p TC domain. This subunit contains a TIM barrel, which is characterized by the $\beta_8\alpha_8$ core motif composed of eight parallel β-strands forming a barrel surrounded by eight α-helices (Banner *et al.*, 1975). The C-terminal truncated HpPyc1$^{1–815}$p contains the complete $\beta_8\alpha_8$ core of the TC domain. However, only very minor amounts of this truncated version of HpPyc1p were observed (Fig. 8b), despite the fact that it was expressed under control of the strong P_{AOX}. Probably, all structural elements in the HpPyc1p TC domain are required to form a stable conformation. This is supported by the observation that several pentapeptide insertions in the TC domain resulted in relatively low protein levels (Fig. 3).

Because deletions in the region between the BC and TC domains also resulted in low levels of HpPyc1p (in HpPyc1$^{512–1175}$p and HpPyc1$^{561–1175}$p), this region of the protein could be part of the highly structured TC domain in eukaryotic Pyc proteins as well. This is also suggested by data obtained by Lim *et al.* (1988), who showed that upon limited proteolysis of *S. cerevisiae* Pyc, a fragment was obtained that represented the TC domain, including the linking region (residues 473–967 in ScPyc, corresponding to HpPyc residues 477–971).

How the central domain of HpPyc1p functions in assisting FAD binding to AO apomonomers is still unknown. Possibly, HpPyc1p binds to newly synthesized AO monomers and stabilizes a conformation that allows FAD binding. Detailed *in vitro* reconstitution experiments may shed further light on this intriguing phenomenon.

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